PATHOGENESIS, DETECTION, PREVENTION AND TREATMENT OF INFECTIOUS DISEASES OF MILITARY IMPORTANCE

Richard B. Hornick, M.D.

Annual Report for 1973

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13. ABSTRACT

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INTRODUCTION

The Division of Infectious Diseases of the University of Maryland School of Medicine continues to maintain a volunteer research ward at the Maryland House of Correction, Jessup, Maryland. The purpose of this facility has been to serve as a base for investigations in volunteers of various uniquely human infectious diseases. These studies have been aimed primarily at the evaluation of various vaccines. In recent years the effort supported by this contract has been directed at enteric infections of military importance. Other studies supported by Department of Defense and National Institutes of Health contracts have integrated very nicely allowing for a broad research effort into enteric infections. Thus, quantitative measurements of effectiveness of oral typhoid and shigella vaccines have been carried out. In addition, attempts have been made to uncover the significance of pathogenesis of and host resistance to infection with the presumed viral agent of "winter vomiting" disease, enterotoxigenic E. coli and penetrating E. coli. Availability of these human models has allowed for cooperative research projects with units such as USAMRIID whose competence in metabolic studies permits wider utilization of their expertise, to explore the role of endotoxin in infectious diseases, and to attempt to define mechanisms of immunity, i.e., cellular versus humoral. The status of these studies will be presented in this report.

A. UTLIZATION OF INMATES AS VOLUNTEERS

In the last 13 years a large number of volunteers have been employed in the course of our investigations. Numerous administrative changes have occurred at the prison which affect our program. The current warden (the fourth throughout our stay at the prison) continues to be cooperative and interested in the program. However, he feels somewhat exposed in his position and decision to allow the studies to continue. Despite our efforts to support his position, such as assurances from the Office of the State Department of Health and the Superintendent of Prisons for Maryland, the Warden remains concerned. Because of this a meeting is planned for late 1974. Numerous state officials will be invited; i.e., State Department of Health, Attorney General, Superintendent of Prisons, as well as other interested individuals including chaplains, representatives of the Civil Liberties Union, sociologists, Department of Defense and NIH administrators and probably inmates. The purpose of this meeting will be to attempt to present the program (again) to this group and to try to attain some feeling of the group as to the future of inmate participation in properly supervised medical research. The new regulatory proposals published in the Federal Register on November 16, 1973, will be presented as the latest form of guidelines that will be followed by our group. We obviously feel this is an important program, unique in its scope and aims and worthy of continuation. By getting a group together to discuss inmate volunteerism, we hope to improve communication so that a better understanding can be achieved between the scientific and public communities.

At the present time inmates at the Maryland House of Correction continue to volunteer for our projects but not in the numbers as previously. One reason for this has been the insistance by the prison officials that only unemployed inmates participate. Those with jobs will lose them if they are hospitalized. They can be on out-patient type of studies (vaccine administration) but will lose their job as noted if admitted. This has cut down the available number of inmates by half, approximately 700 men instead of 1500. Furthermore, there are inmates who feel that volunteering for a study is "not the thing to do", and some of the men relate any study to the Tuskegee Syphilis study. Attempts to improve the communication gap at the prison are underway. No attempt has been made to increase the amount of money paid to inmates (remains at $2/day) or to promise any benefit pertaining to parole or reduction of sentence. We feel strongly that these are unethical considerations and have no place in the recruitment of volunteers. Despite this decrease in the number of volunteers, significant studies have been conducted and those involved in enteric research will be presented here.

B. ORAL TYPHOID VACCINE STUDIES

Reasons for emphasis on oral vaccines to prevent typhoid fever have been enumerated in previous reports. Briefly, this and other enteric infections are caused by organisms that must

gain entrance into the body through the intestinal epithelium. It is obvious that many unknown (specific and non-specific) factors are involved in the resistance to pathogen penetration. Volunteers given identical numbers of S. typhi vary in their responses; some (about 30% following 10^5 organisms) will develop typhoid fever, the remainder either have evidence of infection with a few positive stool cultures, serological responses or low grade temporary fever and the rest have no measurable evidence of having had an encounter with typhoid bacilli. These organisms were swallowed but the subsequent events determining their fate are unknown. Hopefully, the factors involved in protecting these men can be identified. Typhoid carriers have large numbers of organisms passing through the gut which do not cause reinfection in the host. In such cases typhoid bacilli are confined to the lumen of the gut by unknown defense mechanisms.

The earlier studies with oral vaccines for typhoid fever demonstrated that killed organisms given by mouth appeared to induce some bactericidal activity in the gut since cultures of stools for typhoid bacilli revealed fewer positives in the vaccine recipient group compared to controls but no change in incidence of disease following challenge. Subsequent investigations of live attenuated strains have employed a streptomycin-dependent strain (SmD) as well as an epimeraseless strain. These strains have given evidence of being potentially effective oral vaccines. The dose of each vaccine strain has been empirically chosen, the largest numberof bacilli that can be given safely and which can be readily concocted. Thus, each dose has contained approximately 10^{10} organisms. Doses have been given biweekly for four weeks. Fresh cultures of the streptomycin-dependent strain gave excellent results both in terms of protection against disease and in reducing the shedding of pathogens in stools. However, the initial attempt at lyophilization of this strain resulted in an ineffectual vaccine. Several technical problems in preparing the lyophylized vaccine were thought to be responsible for the poor results. A repeat lyophilization was done and the vaccine was administered to volunteers in the usual large doses but without concurrent streptomycin. This method (i.e., without streptomycin) is thought to be the manner in which the vaccine should be administered in the field. However, in the volunteers receiving this vaccine, attack rate was similar to that seen in controls. It is quite obvious that either much larger and repeated doses of vaccine must be given by mouth or else streptomycin must be administered simultaneously. This latter alternative is not very attractive because of the fear of selecting resistant strains of E. coli and other aerobic organisms in the gut flora. In addition, there is the remote risk of hypersensitivity reaction to the streptomycin. Additional studies are now underway to determine whether lyophilized vaccine plus streptomycin will induce protection at a level equivalent to the fresh strain. (Note: In a subsequent section is listed the results of a mouse protection test comparing fresh versus lyophilized SmD vaccines.)

The preliminary data gathered with the epimeraseless strain is very promising. No adverse reactions have occurred although a few instances of mild diarrhea have been recorded. The leve of resistance induced with this strain has been superb; one case of typhoid out of

12 challenged volunteers compared to a 43% attack rate in the controls. A group of 57 volunteers have recently been vaccinated with repeated doses of this strain which had been lyophilized. Challenge is planned for mid-January 1974. If this vaccine induces immunity as previously, it will be ready for field testing.

The number and size of doses of attenuated strains needed to induce immunity are unknown and difficult to ascertain. As noted, our approach has been empirical since no measure of gut immunity is available. In the volunteer population several approaches have been made to ascertain the nature of induced immunity. These studies have centered mainly on the role of local antibodies and cellular immunity. These studies are discussed below. Nevertheless, if these vaccines prove to be effective in the volunteer population, a non-endemic group in terms of exposure to typhoid fever, they should be even more effective in areas of the world where typhoid is common. Orally administered vaccines should complement the naturally acquired immunity and should be very beneficial in children, a group with a high attack rate of typhoid fever in endemic areas. These children can have an attenuated vaccine substituted for the more virulent orally ingested typhoid bacilli that either induce immunity or cause active disease. A field trial with these vaccines is planned and application for funds to conduct such a study has been submitted to the U.S.A.I.D. program. Hopefully, if funded, the volunteer studies would complement the investigations conducted in the field. The proposed site for the field trial is Peru where typhoid fever is a very common disease. The control vaccines in these studies would be an attenuated shigella vaccine.

C. MOUSE POTENCY TEST OF STREPTOMYCIN-DEPENDENT TYPHOID VACCINE

In an effort to determine whether or not lyophilization had in any way altered the antigenic content of the streptomycin-dependent typhoid vaccine, standard mouse potency tests were conducted. These tests would not measure the factors which may be responsible for live vaccine efficacy as multiplication and persistency in vivo but would measure immunogen content as they do for parenteral vaccines.

On September 25, 1973, fresh streptomycin-dependent typhoid vaccine was prepared and diluted to the same turbidity as a freshly rehydrated sample of lyophilized streptomycin-dependent vaccine. Replicate plate counts of these two vaccines showed a viable colony forming unit count to be 2.58×10^{10}/ml for the lyophilized vaccine and 1.24×10^{10}/ml for the freshly prepared vaccine.

Mice were vaccinated with these vaccines as proscribed by NIH protocol for testing typhoid vaccine potency. Five-fold dilutions beginning at undiluted vaccine were administered intraperitoneally in 0.5 ml doses to groups of mice. At the same time five-fold dilutions of NIH Standard Vaccine were given similarly beginning at a 1:10 dilution.

Both the lyophilized and fresh experimental vaccines were toxic to the mice at each dilution; 20 of 20 mice died within 24 hours of inoculation in each vaccine group. The 1:5 dilution of each vaccine was also toxic; 17 of 20 inoculated with the lyophilized vaccine died within 48 hours and 16 of 20 mice given the fresh vaccine died within 24 hours. Late sporadic and non-specific deaths occurred in the two week period before challenge.

On October 9, 1973, 14 days after vaccine administration, the mice were challenged IP with 0.5 ml of virulent S. typhosa Ty2v; each 0.5 ml contained 1250 organisms in 0.5% mucin. The following table presents the results of challenge:

	Streptomycin-Dependent Vaccine		NIH Control Vaccine	
Dilution	Fresh	Lyophilized	Dilution	
1:5	0/4*	0/2*	1:10	2/19*
1:25	4/16	4/18	1:50	3/19
1:125	0/19	4/20	1:250	4/17
1:625	2/19	1/17	1:1250	5/19
1:3125	2/17	2/20	1:6250	9/17

Controls:	1250 organisms	20/30*
	125 "	11/19
	12.5 "	8/20
	1.5 "	4/20

*Deaths/total inoculated

From the above data we were unable to show any differences between the protective capacity of the lyophilized and fresh vaccine for mice receiving the vaccine IP and tested by the standard method as defined for killed parenteral vaccines.

Tests run to determine whether the addition of 0.8 Gm sodium bicarbonate and milk to the vaccine just prior to its administration demonstrated no effect of these materials on the viability of the organisms.

D. ANTIBIOTIC RESISTANT TYPHOID STRAINS

In the spring of 1972, an epidemic of typhoid fever occurred in Mexico, centering mainly around Mexico City. This epidemic was thought to be caused by strains of typhoid bacilli resistant to chloramphenicol. However, the physicians in Mexico continued to use this drug because of its long history of reliability in treating patients with typhoid fever. Also reports were received from Mexico indicating that at least seven lots of chloramphenicol of foreign manufacture failed to achieve U.S. standards on pharmacological testing. The situation concerning therapy in these patients was chaotic because of the numerous antibiotics that were employed to treat these patients. The selection of an antibiotic once chloramphenicol failed to work was based on in vitro sensitivity testing, a test which does not correlate well with in vivo activity. Thus, our group in cooperation with physicians at the IMAN Hospital in Mexico City set up a prospective trial of chloramphenicol and ampicillin to treat patients. Appropriate safeguards were built into the protocol to insure the safety of the patient that may receive chloramphenicol and who was infected with a chloramphenicol resistant strain. (Ninety percent of patients treated in this hospital were infected with the resistant strain.) Fifteen patients were treated with certified chloramphenicol and 18 with ampicillin. Eleven of the 15 were retreated with ampicillin and three of these 11 had chloramphenicol discontinued because of complications and then were switched to ampicillin. Four of 15 patients apparently responded while on chloramphenicol despite infection with a resistant strain. All of the ampicillin treated group had a satisfactory therapeutic response although 30% had a drug rash. Bacteremia disappeared in 1.5 days in the group of patients treated with ampicillin while it took almost 5 days in the patients treated with chloramphenicol. Obviously, in vitro studies indicating resistance to chloramphenicol are valid and such studies are needed in patients acquiring this disease in areas of the world where typhoid strains have become resistant to chloramphenicol.

In 1973, another therapeutic trial was conducted to evaluate the affectiveness of trimethoprim/sulfamethoxazole combination versus a new analogue of ampicillin, amoxycillin. This latter drug can be given by mouth and gives blood levels of active drug 2 - 3 times greater than ampicillin. The concern for conducting such an evaluation stemmed from the isolation of 27 strains of typhoid bacilli in 1972 that were resistant to ampicillin. Some of these strains were isolated from blood or bone marrow (not just stool specimens) where the likelihood of R transfer factor assimilation could occur. In the 1973 trial, both drugs were found to be very affective in treating patients infected with chloramphenicol-resistant strains. It was also found that the sulfa drug had no role in treating these patients since the strains were resistant to it. In sensitive strains (chloramphenicol and sulfa) there did appear to be an enhanced therapeutic effect. Some toxic side effects were noted with the combination drug, especially thrombocytopenia, and sufficient to cause discontinuation of the drugs. Thus, these two antibiotics are useful in treating patients with disease caused by chloramphenicol resistant strains. If ampicillin resistance emerges as a common event (this probability seems likely) the combination of trimethoprim/sulfamethoxazole (Septrim, Bactrin) appears to be the only therapeutic agent with any promise. Fortunately, during the studies in 1973, the incidence of chloramphenicol

resistant strains fell from 90% in 1972 to 63%. Perhaps this disease was mediated by avoidance of chloramphenicol in treating febrile patients less, lowering the antibiotic selection pressure.

Also in 1973, another new area of the world reported the occurrence of chloramphenicol resistant typhoid strains, South Vietnam. Thus, these strains have been reported from the Middle East, Southeast Asia, Central and South America. In this country cases have been reported in tourists from Mexico, but no secondary cases have occurred. Twenty-five percent of the strains isolated in the U.S.A. in 1973 have been resistant to chloramphenicol. This evidence emphasizes the need to continuously monitor the antibiotic sensitivity of typhoid strains isolated around the world.

E. INVESTIGATIONS OF IMMUNE MECHANISMS IN TYPHOID FEVER

In previous reports we have described experiments designed to assess the possible role of cellular immunity in host defense mechanisms against typhoid fever. In these studies the lymphocyte transformation reaction (as monitored by the uptake of tritiated thymidine in newly synthesized DNA) was used to study the response of cultured lymphocytes from volunteers given oral typhoid vaccines. The results obtained when S. typhosa lipopolysaccharide (LPS-Difco) was employed as the in vitro stimulatory antigen were presented and discussed in the Annual Progress Report of August 1972. We now wish to present data obtained in two subsequent studies; one in which both LPS and a sonicate of the Quailes strain were used as in vitro antigens, and another in which a subcellular protein fraction derived from living S. typhosa 0901 organisms was employed to stimulate lymphocytes in vitro. In both studies the lymphocyte donors were volunteers who had received living, oral typhoid vaccines.

Table 1 records the pre- and post-vaccination lymphocyte responses to LPS and the Quailes sonicate. Each antigen was tested at three concentrations: LPS at 1, 10 and 100 ug per culture and the sonicate at concentrations corresponding to 7×10^3, 7×10^4 and 7×10^5 organisms per culture. Stimulation ratios >2 were considered significant, and the highest ratio obtained with either antigen is reported in the table with the corresponding antigen concentration in parentheses.

Of the sixteen volunteers whose pre-vaccination responses to both antigens were studied (Table 7, Annual Report 1972), only ten completed the vaccination schedule, and of these, only five were challenged (Table 1). Despite this attrition in our experimental population, we think the following observations deserve mention.

1. Oral vaccination was not necessarily accompanied by increased in vitro lymphocytes responses to either or both antigens.

2. Since stimulation indices for both antigens paralleled each other roughly in 14/19 instances, a question arises concerning the nature of the lymphocyte stimulating substance in the sonicate. When our lot of LPS was compared with the sonicate for endotoxin activity by the Limulus assay, 1 ug of LPS corresponded in activity to 7×10^6 organisms of sonicate. Thus, on this basis, it seems unlikely that LPS was responsible for the responses observed with the sonicate. Moreover, in three instances (R.M., J.H., and H.A.) responses with the sonicate were observed in the absence of any to LPS and the converse is true with G.F. and J.B.

3. Despite the small number of challenged volunteers, lymphocyte stimulation by either of these antigens was not necessarily associated with protection. On this basis, we conclude that neither LPS nor the sonicate are effective antigens for the purpose of evaluating an individual's immune status with respect to challenge with S. typhosa. Indeed, the significance of in vitro lymphocyte responses to these antigens remains elusive.

The most recent antigenic preparations derived from S. typhosa to be tested for its ability to detect cellular immunity in typhoid vaccinees was prepared according to the method of Smith and Bigley (Infect. Immunity 6:377-383, 1972). It corresponds to their "NP fraction" derived by passing viable S. typhosa three times through a French pressure cell, precipitating the whole cell RNA fraction with ethanol and then precipitating the protein from this material with two volumes of saturated $(NH_4)_2SO_4$ solution. By disc electrophoresis the resulting preparation was observed to contain eleven protein bands. No precipitin bands were discerned by double diffusion in agar with antisera raised against S. typhosa strains 0901 and Ty2.

When this material was used to test its stimulatory activity on cultured lymphocytes obtained from vaccinated and unvaccinated volunteers, a stimulation index less than one was usually obtained (Tables 2 and 3). In fact, no stimulation was observed in lymphocyte cultures from two recent typhoid convalescents (Figure 1) at any concentration of protein through 110 ug.

Viability studies suggest this material is toxic. Lymphocytes cultured in the presence of up to 100 ug of LPS or up to the equivalent of 7×10^7 S. typhosa organisms, as represented by the Quailes sonicate, show 90 percent or better viability after 5 days of culture. At the lower concentrations of the sub-cellular protein fraction, 80-85 percent viability was observed after 5 days and at higher concentrations, only 50-60 percent of the cells were viable. Thus, failure to observe stimulation with this material may be related to its toxicity.

Table 3 tabulates the responses of volunteers whose lymphocyte cultures were set up with the sub-cellular S. typhosa protein fraction four days after challenge. Initially, this was done with the hope that challenge might increase the concentration of specifically sensitized circulating lymphocytes and thus provide a better base for correlating stimulation with the outcome of challenge. This was not possible since no stimulation occurred, but we wish

to draw attention to the low PHA stimulation ratios obtained in this group compared with those recorded in Tables 1 and 2. Since these results were not anticipated, their significance is questionable in the absence of appropriate controls. They suggest, however, that challenge with S. typhosa may have depressed normal T-cell responses to PHA. We have designed a controlled experiment in order to study the validity of this hypothesis at the time of the next challenge.

In summary, we conclude that if cellular immunity plays a role in host defense mechanisms against typhoid fever, the method and/or the antigens so far employed have failed to provide definitive information to this effect.

The Effect of Endotoxin on the PPD Induced Stimulation of Cultured Lymphocytes from PPD Sensitive Donors:

Because of the presence of a small amount of endotoxin in the S. typhosa (Quailes) sonicate used for the lymphocyte transformation studies (vide supra), we decided to study the effect, if any, of endotoxin on the in vitro stimulation of T-lymphocytes by an appropriate antigen. Because of the availability of PPD sensitive donors, we chose to study the effect of endotoxin on PPD induced lymphocyte blastogenesis.

Table 4 summarizes the initial experiment in which 100 ug of S. typhosa LPS was present in cultures containing phytohemagglutinin (PHA), pokeweed mitogen (PWM), and varying concentrations of PPD (NIH Research Material, Lederle). No effect of LPS was noted on PHA stimulated cultures and the effect on PWM cultures was variable. However, markedly decreased counts were obtained in the PPD stimulated cultures.

Next, the LPS was titrated to determine the effect of varying concentrations of endotoxin on lymphocytes stimulated at a single concentration of PPD. Figure 2 represents data from three experiments utilizing cells from a single PPD sensitive individual. A straight-line relationship was not observed. Rather, little difference in the effect of LPS was noted at concentrations ranging from 10^{-2} to 10^{2} ug per culture. At 10^{-6} ug per culture, the lowest concentration tested, only 74 percent of the control counts were obtained.

The next experiments were set up to determine whether a similar effect could be obtained with smooth LPS from another organism and from LPS derived from a rough strain. Table 5 summarizes these results which suggest that the ability of LPS to decrease ^{3}H-thymidine uptake by PPD stimulated lymphocytes is associated with core lipopolysaccharide rather than with the terminal sugar residues.

We intend to repeat these experiments and extend our observations in an effort to determine how LPS, a thymus independent antigen, acts to decrease the potential stimulatory activity of a T-cell dependent antigen in this system.

Does Endotoxin Affect the Induction and/or Elicitation of Delayed Hypersensitivity Reactions in vivo?

Our approach to this important problem was described in the contract renewal submitted earlier this year. The work is underway, and a preliminary report will be filed at a later date.

TABLE 1

IN VITRO RESPONSES TO TYPHOID ANTIGENS OF LYMPHOCYTES DRAWN FROM VOLUNTEERS BEFORE AND AFTER ORAL VACCINATION WITH LIVING TYPHOID VACCINES

SUBJECT & VACCINE	PRE/POST VACCINATION	ANTi-O TITER	cpm STIMULATED / cpm CONTROL			OUTCOME OF CHALLENGE
			PHA	LPS*	SONICATE*	
R.S. Ty21a	Pre	40	30	<1	<1	
	Post	40	86	2.7(100)	2.3 (7×10^5)	No disease
F.B. Ty21a	Pre	<20	48	<1	<1	
	Post	<20	32	<1	<1	No disease
J.M. Ty21a	Pre	<20	204	<1	<1	
	Post	<20	300	N.D.	<1	No disease
G.F. Ty21a	Pre	40	375	2.4 (1)	1.4	
	Post	40	112	1.0	1.0	Disease
R.U. SmD	Pre	80	27	1.4	1.1	
	Post	80	63	4.9 (100)	2.0 (7×10^3)	Disease
R.M. Ty21a	Pre	<20	30	1.3	2.1 (7×10^4)	
	Post	<20	86	1.5	1.8	Not challenged
J.H. Ty21a	Pre	<20	77	1.7	2.4 (7×10^5)	
	Post	<20	145	2.9 (100)	2.2 (7×10^4)	Not challenged
J.J. SmD	Pre	<20	40	<1	<1	
	Post	<20	400	2.1 (100)	2.6 (7×10^3)	Not challenged
H.A. Ty21a	Pre	<20	68	1.4	2.0 (7.10^4)	
	Post	<20	32	<1	<1	Not challenged
J.B. Ty21a	Pre	<20	31	<1	<1	
	Post	<20	18	4.8 (100)	1.4	Not challenged

* Numbers in parentheses indicate concentration of stimulating antigen in culture expressed as ug LPS and number of organisms in sonicate.

TABLE 2

IN VITRO RESPONSES TO A SUBCELLULAR PROTEIN FRACTION FROM S. TYPHOSA OF LYMPHOCYTES FROM VACCINATED AND UNVACCINATED VOLUNTEERS

VOLUNTEER	cpm STIMULATED / cpm CONTROL			DISEASE
Tested 4 days before challenge	PHA	22 ug STP	66 ug STP	
C.A. (v)	19	0.6	0.7	0
R.H. (v)	16	0.5	0.5	0
H.H. (v)	8	1.2	1.0	0
R.C. (v)	45	0.8	0.7	0
C.J. (v)	60	0.6	0.6	+
A.S. (v)	22	0.3	0.2	0
M.McD.(v)	5	0.8	1.2	0
K.P. (v)	46	0.7	0.6	0
W.W.(v)	30	0.2	0.2	0
M S. (v)	65	1.3	1.5	0
H.F. (v)	12	1.0	0.7	+
A.J. (c)	57	0.7	0.7	0
G.M.(c)	36	0.7	0.7	0
G.R. (c)	49	0.5	0.5	0
D.G. (c)	18	0.7	0.6	0
A.H. (c)	70	0.6	0.4	+

(c) unvaccinated controls (v) vaccinees

TABLE 3

IN VITRO RESPONSES TO A SUBCELLULAR PROTEIN FRACTION FROM S. TYPHOSA OF LYMPHOCYTES SET UP FOUR DAYS AFTER CHALLENGE

VOLUNTEER Tested 4 days after challenge	cpm STIMULATED / cpm CONTROL			DISEASE
	PHA	27.6 ug STP	82.8 ug STP	
M.W. (v)	5	0.9	0.9	0
T.J. (v)	26	0.5	0.5	0
D.M. (v)	5	0.9	1.8	0
J.W. (v)	14	1.0	0.5	0
D.L. (v)	14	0.9	0.7	+
G.R. (c)	1	0.09	0.08	+
M.M. (c)	11	1.1	1.5	0

(c) unvaccinated controls (v) vaccinees

TABLE 4

THE EFFECT OF S. TYPHOSA LPS ON LYMPHOCYTE STIMULATION IN VITRO BY MITOGENS AND PPD

SUBJECT	STIMULANT	CONTROLS dpm ($\times 10^{-3}$)	+100 ug LPS dpm ($\times 10^{-3}$)	% dpm in presence of LPS
Teal	0	9.6	17.4	-
	PHA	560	921	164
	PWM	687	57	8
	PPD, 5 ug	38	16	41
	PPD, 10 ug	25	12	48
	PPD, 20 ug	25	13	52
Powers	0	17	29	-
	PHA	499	581	116
	PWM	468	344	73
	PPD, 5 ug	537	29	5
	PPD, 10 ug	267	47	14
	PPD, 20 ug	203	52	25
Prevette	0	34	12	-
	PHA	1,140	1,116	98
	PWM	585	593	100
	PPD, 5 ug	163	34	21
	PPD, 10 ug	296	49	16
	PPD, 20 ug	165	62	37

TABLE 5

THE EFFECT OF "SMOOTH" AND "ROUGH" LIPOPOLYSACCHARIDES ON LYMPHOCYTE STIMULATION IN VITRO BY PPD

EXPERIMENT	LPS and SOURCE ug/culture		dpm ($\times 10^{-3}$) w/o PPD	dpm ($\times 10^{-3}$) w/10 ug PPD	dpm LPS + PPD / dpm PPD
1	0		5.6	60.0	-
	S. typhosa (Difco)	10^{0}	5.3	27.5	45
		10^{-1}	3.8	27.2	45
		10^{-2}	5.2	29.0	48
	E. coli (Difco)	10^{1}	5.5	30.4	50
		10^{-1}	5.4	29.3	49
		10^{-2}	5.0	32.5	54
2	0		6.6	79.9	-
	S. typhosa (Difco)	10^{-2}	8.0	28.7	36
		10^{-4}	5.5	55.9	70
		10^{-6}	5.6	58.9	73
	S. typhimurium (rough mutant)	10^{-1}	7.3	25.0	31
		10^{-3}	7.0	29.0	36

Figure 1 In vitro Lymphocyte Responses of Typhoid Convalescents to a Subcellular Protein Fraction of S. typhosa

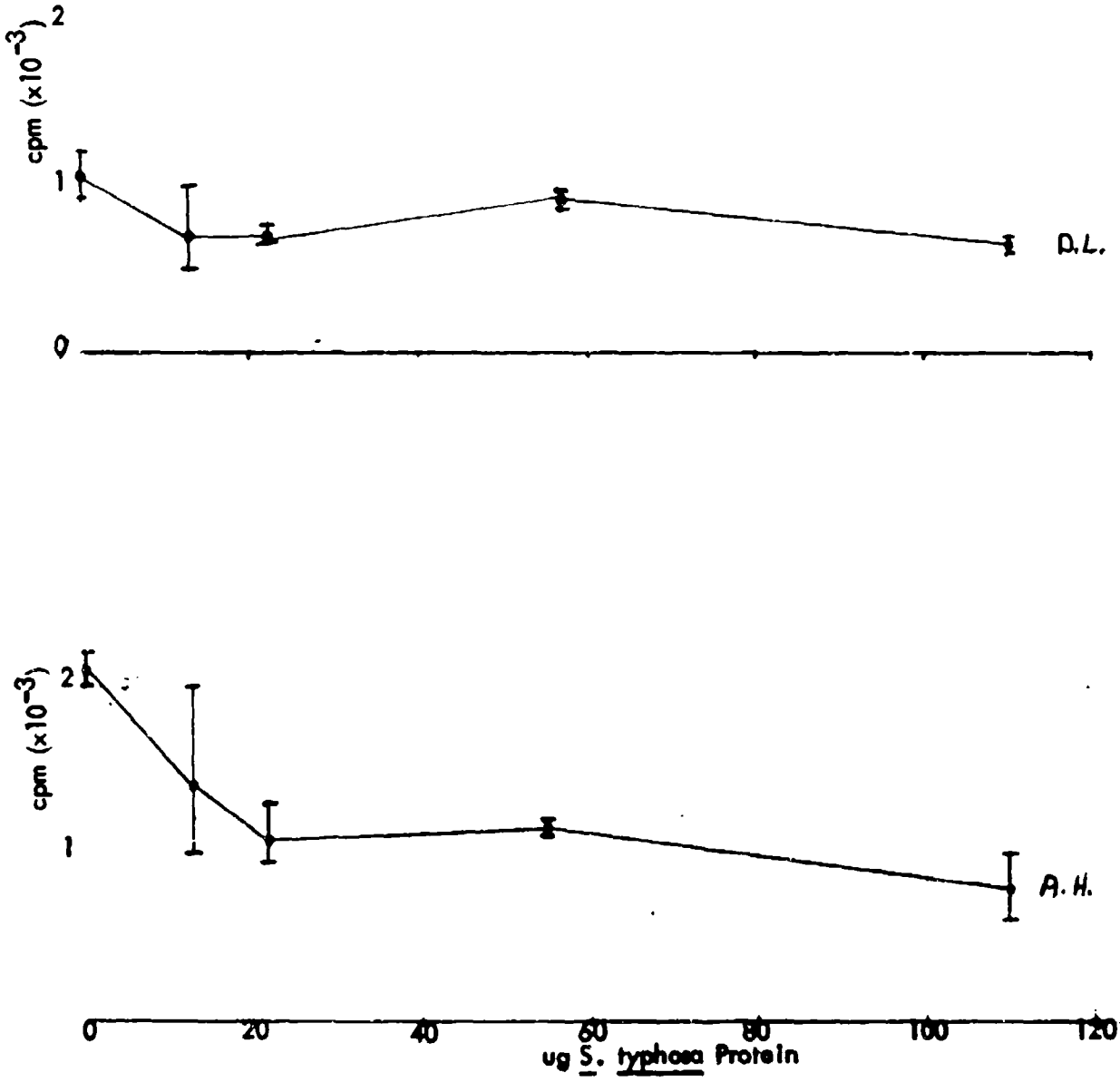
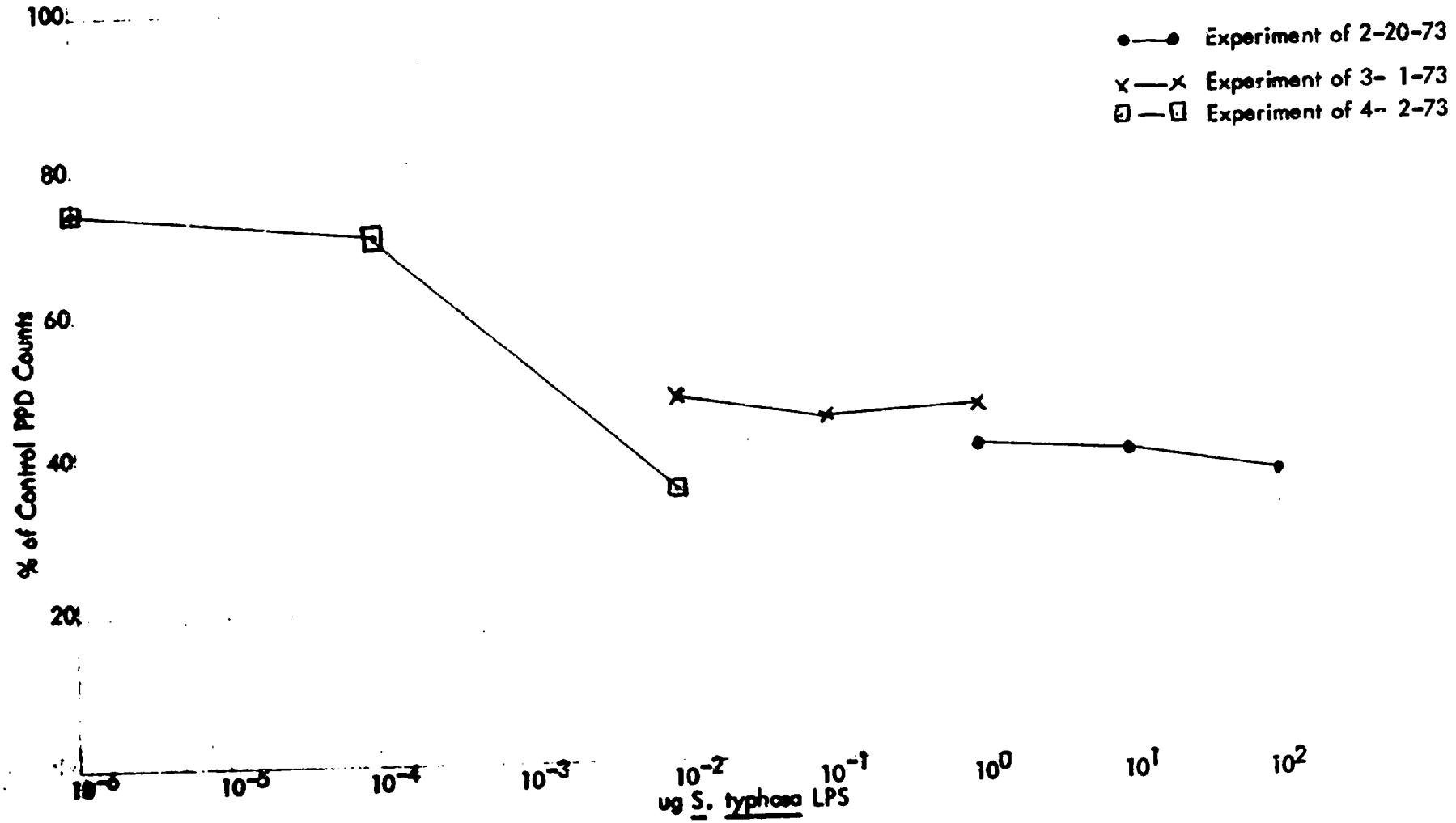


Figure 2. The Effect of Varying Concentrations of S. typhosa LPS on the Uptake of ^{3}H-thymidine by the Lymphocytes of a Single PPD Sensitive Donor Stimulated with 10 ug of PPD



REFERENCES:

Hornick, R.B.: Salmonella Infections - Newer Perspectives of an Old Infection. Presented as the Jeremiah Metzger Lecture, American Clinical & Climatological Association, (October 1973), Trans. Amer. Clin. Climatol. Assoc., In press.

Levine, M.M., DuPont, H.L., Formal, S.B., et al: Pathogenesis of Shigella Dysenteriae I (Shiga) Dysentery. J. Infect. Dis. 127:261-270, 1973.

Levine, M.M., DuPont, H.L., Hornick, R.B., et al: Long Term Shigella Carrier State. New Engl. J. Med. 288:1169-1171, 1973.

DuPont, H.L. and Hornick, R.B.: Clinical Approach to Infectious Diarrheas. Medicine 52:265-270, 1973.

DuPont, H.L., Hornick, R.B., Dawkins, A.T., et al: Rocky Mountain Spotted Fever: A Comparative Study of the Active Immunity Induced by Inactivated and Viable Pathogenic Rickettsia rickettsii. J. Infect. Dis.

Togo, Y., Schwartz, A.R., Hornick, R.B.: Antiviral Effect of DIQA in Experimental Human Rhinovirus Infection. Antimicrob. Ag. Chemother. (December 1973) In press.

Schwartz, A.R., Togo, Y., Hornick, R.B., et al: Evaluation of the Efficacy of Ascorbic Acid in Prophylaxis of Induced Rhinovirus 44 Infection in Man. J. Infect. Dis. 128:500-505, (October), 1973.

Togo, Y., Schwartz, A.R., Hornick, R.B.: Failure of a 3-Substituted Triazinoindole in the Prevention of Experimental Human Rhinovirus Infection. Chemotherapy (Karger) 18:12-26, (January), 1973.

Togo, Y.: In Vitro Effect of Virazole Against Influenza Viruses. Antimicrob. Ag. Chemother. (December)1973, In Press.

Included also with this Annual Report are copies of two papers resulting directly from support by this Army contract.

HUMAN LEUKOCYTE MIGRATION INHIBITION: AN ASSAY FOR ENDOTOXEMIA*

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We thank Dr. Lawrence Rothfield, University of Connecticut School of Medicine, who supplied the S. typhimurium endotoxin; and the patients and staff of the Hospital de Enfermadades Infecciosas, Santiago, Chile for their gracious cooperation and assistance.

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ABSTRACT

The ability of endotoxins from gram-negative bacteria to inhibit human leukocyte migration (HLM) has been carefully quantitated. As little as 0.05 ng/ml of highly purified endotoxin significantly inhibited human leukocyte migration (HLM) in a capillary tube system. Additional ten-fold increases in endotoxin concentration produced further significant increments in this depression. Utilizing this phenomenon, an assay was developed which would detect endotoxin in concentrations as low as 0.1 ng endotoxin per ml blood. Bioassays were performed on 13 highly febrile patients with typhoid fever. Criteria for detectable endotoxemia were not fulfilled in any of these patients. The ability of endotoxin to inhibit HLM can provide a useful system for investigations of endotoxemia.

Migration of human leukocytes is inhibited in vitro by bacterial endotoxin (1, 2); however, the extreme sensitivity of this phenomenon does not appear to be fully appreciated. The present studies describe the dose-response relationships of this endotoxin-leukocyte interaction, demonstrate that human leukocyte migration (HLM) is inhibited by sub-nanogram per ml concentrations of endotoxin, and indicate that this response can provide a basis for a sensitive assay for endotoxemia.

MATERIALS AND METHODS

All glassware and pipettes were treated by multiple rinsings with distilled water, and heated overnight at 200°C in a dry air oven to eliminate extraneous pyrogens. Syringes and needles were sterile, non-pyrogenic, and disposagle (Becton, Dickinson, and Co., Rutherford, N. J.).

Preparation of assay suspension. Healthy, fasting donors were used throughout to provide serum and leukocytes. For any given study, and for the typhoid endotoxemia studies, serum and leukocytes from a single donor were employed. Serum was prepared weekly and held at 4°C until use. Leukocyte suspensions were prepared daily, refrigerated, and used within 4 hours. For this purpose, 10 ml venous blood was transferred to a glass test tube containing 0.2 ml (200 U) sterile, non-pyrogenic aqueous heparin without preservative (Mediquest Division, Electrodynamics, Inc., Baltimore, Md.). After gentle mixing, 40 ml sterile, non-pyrogenic 0.9% saline was added and the mixture centrifuged 500 g for 5 minutes at 6°C. The supernate was discarded and after another such washing the resulting cell sediment was gently but thoroughly mixed and used as the leukocyte source.

Migration technique. HLM was measured by the method of Ketchel and Favour (3) using 32 x 0.8 mm microhematocrit capillary tubes (Drummond Scientific Co, Broomall, Pa.). Varying concentrations of endotoxin in saline (or plasma) were added to aliquots of the washed blood cell sediment suspended in isologous serum in iced test tubes to yield a final ratio by volume of 1:10:9 (endotoxin:blood cells: serum). Each resulting suspension was used to fill one set of 26 microhematocrit tubes approximately two-thirds full. The bottoms of the microhematocrit tubes were sealed with clay (Clay-Adams, Inc., New York, N.Y.) and held in an ice bath until all sets were filled. Then all sets, each containing a different concentration of endotoxin, were simultnaeously centrifuged 1,000 g for 5 minutes at 6 °C. The spun tubes were held in an ice bath while each set was checked microscopically for uniform buffy coat packing. Tubes with leukocyte clumping or adherence to walls were discarded. Sixteen acceptable tubes from each set were then taped side by side to a glass slide and incubated vertically for 2 hours in a water bath at 40 °C to promote maximum migration (4, 5). The distance traversed by the advancing cells from the erythrocyte-buffy coat interface was then measured using an ocular micrometer. The 10 most advanced cells in each hematocrit tube were excluded to obtain a more uniform advancing front. The mean migration for each set was calculated and expressed as percent of control migration (migration index).

RESULTS

Dose-response relationship. One-tenth ml of varying concentrations of a Westphal-extracted Salmonella typhimurium endotoxin, suspended in sterile, non-pyrogenic 0.9% saline, was cdded to 1.9 ml of a mixture of washed blood cells (1.0 ml) suspended in isologous serum (0.9 ml) to provide a series of graded endotoxin concentrations; 0.1 ml sterile, non-pyrogenic

0.9% saline without endotoxin was added to control tubes. Typical resulting 2 hr migrations are illustrated in figure 1. Significant HLM depression was evoked by endotoxin concentrations as low as 0.05 ng/ml. Additional logarithmic increases in endotoxin concentration resulted in significant stepwise depressions of HLM.

Subsequent studies were performed to determine if endotoxin added to whole blood could be detected in the resultant plasma. Varying concentrations of S. typhimurium endotoxin were added to iced tubes containing 60 U of heparin and 2.0 ml aliquots of venous blood from fasting healthy donors. Control tubes contained sterile, non-pyrogenic 0.9% saline in place of exogenous endotoxin. Plasma was immediately prepared from these bloods by 500 g centrifugation for 5 min at 6°C. The plasma to be tested for endotoxin was then added to a standard washed blood cell-serum suspension to produce final plasma:blood cell: serum ratios by volume of 1:10:9, respectively. (It is emphasized that the blood cell-serum suspension was alwyas prepared from the same donor, whereas the plasmas to be assayed for endotoxin content were prepared from a variety of healthy donors.) Migration inhibition produced by the various plasmas is illustrated in figure 2. Plasmas from donors A, B, and C, possessing known exogenous endotoxin concentrations as low as 0.2 ng/ml donor blood, produced significant depression of HLM. In donor D, 0.2 ng endotoxin/ml blood did not produce significant depression in the assay of fresh D plasma; however, 2 frozen plasma aliquots assayed 3 and 4 weeks later did demonstrate significant depression at this concentration. Thus, exogenous endotoxin added to whole blood at concentrations of 0.2 ng/ml produced significant depression of HLM in five of six assays. Moreover, significant HLM depression was detected in all assays at endotoxin concentrations of 2.0 ng/ml blood; and this activity

persisted in frozen plasma for at least 4 weeks.

As shown in figure 2, plasmas obtained from whole blood to which ten-fold increments of endotoxin were added evoked progressive significant depressions of HLM similar to that of the endotoxin increments in saline, figure 1. The sensitivity of the HLM inhibition dose-response relationship was such that ten-fold increases in endotoxin concentration were required to detect such progressive significant inhibition; two-fold increases in endotoxin concentration failed to further depress HLM by amounts which would be accepted as statistically significant ($p < 0.05$), figure 3.

The above studies were all conducted with a Westphal preparation of S. typhimurium endotoxin. To test whether a different endotoxin would elicit similar dose-responses, varying concentrations of an S. typhosa Boivin extracted endotoxin (Difco Laboratories, Inc., Detroit, MI) suspended in 0.9% saline were added to standard washed blood cell-serum suspensions in a final ratio by volume of 1:10:9. This second endotoxin acted similarly to the S. typhimurium endotoxin although it was not as potent an inhibitor of HLM, figure 4A. Indeed, the potency of the endotoxins in HLM inhibition paralleled their pyrogenic activity in acclimatized rabbits; .01 ug/kg of S. typhimurium endotoxin produced fevers equal in magnitude to 0.1 ug/kg of the S. typhosa endotoxin. Moreover, when the two endotoxins were combined at concentrations eliciting similar HLM inhibitory activity, no further significant depression occurred from that produced by either endotoxin acting alone, figure 4B. This paralleled the minimal effect seen when the concentration of either endotoxin alone was doubled. Thus, different endotoxins do not significantly enhance or inhibit their intrinsic HLM inhibitory activity when combined.

Clinical assay. The above findings indicated that HLM could be used for the detection of endotoxin in human plasma. However, different human plasma vary in their ability to support HLM (3). Hence any reliable assay for endotoxemia must minimize non-specific HLM depressant effects of various plasmas, as well ad distinguish these effects from the HLM depression secondary to endotoxin. This was accomplished by exploiting the following three principles: 1) the various plasmas to be tested were always assayed by addition to a single standard healthy donor blood cell-serum suspension. The effects of known concentrations of endotoxin in plasma in this standard system have already been characterized, figures 2 and 3. Moreover, by diluting the test plasma (0.1 ml) in a large volume of the normal blood cell-serum suspension (1.9 ml) any non-specific depressing effect of the test plasma would be minimized. 2) Plasma from a test blood which evokes significant HLM depression consequent to the presence of endotoxin should not evoke further significant increments in HLM depression when a minimal concentration of exogenous endotoxin, which in itself is just capable of evoking significant HLM inhibition, is added to it. This tenet is derived from the finding that, while ten-fold increases in endotoxin concentration produce significant increments in HLM depression, merely doubling the endotoxin concentration does not significantly augment HLM depression. As depicted in figures 2 and 2, the lowest endotoxin concentration capable of consistently evoking detectable HLM inhibition is in the range of 0.1-1.0 ng/ml blood, and since a patient must have an endogenous endotoxin concentration of at least this magnitude to elicit significant HLM depression, addition of 0.1-1.0 ng/ml blood of exogenous endotoxin should not significantly further depress HLM. 3) All clinical acute phase plasma specimens interpreted as being positive for endogenous endotoxin by criteria one

(significant depression of HLM by plasma) and two (no further significant depression of HLM upon addition of 0.1-1.0 ng/ml exogenous endotoxin) should have a matched control performed in which it is demonstrated that 0.1-1.0 ng/ml exogenous endotoxin added to the convalescent blood does significantly augment the HLM depressant activity of the plasma.

Using the above criteria, a group of patients with naturally acquired typhoid fever were assayed for endotoxemia. Since the most highly febrile patients would be the more likely to have endotoxemia, patients selected were young adults with oral temperatures of 40°C or greater and blood or stool cultures positive for S. typhosa. These patients had no other underlying diseases and were not receiving steroids (6). Duration of illness ranged from 7 - 21 days. Some patients were receiving one of four antibiotic regimens consisting of oral chloramphenicol, chloramphenicol succinate IM, oral ampicillin, or an oral trimethoprim-sulfamethoxazole combination. The findings in these patients with respect to HLM depression were not different from those patients not on these drugs. Six ml of venous blood was obtained from each patient and 2.0 ml aliquots were immediately added to three iced, heparinized tubes containing non-pyrogenic 0.9% saline or the S. typhimurium endotoxin to produce concentrations of 0.0, 0.1, and 1.0 ng exogenous endotoxin per ml blood. Plasma was obtained by centrifugation in iced buckets and held overnight at 4°C. Each set of three plasmas was then assayed by addition to standard blood cell-serum suspensions obtained from a single healthy donor to yield a ratio by volume of 1:10:9 (test plasma:blood cells: serum). Controls consisted of the standard blood cell-serum suspension alone.

Thirteen patients who fulfilled the above criteria for severe typhoid fever were assayed. On the basis of the HLM response patterns, the data could be divided into two

groups. The first group consisted of three patients whose plasmas elicited no HLM depression when compared with controls, figure 5; moreover, the plasmas evoked significant depression of HLM when 0.1-1.0 ng/ml exogenous endotoxin was added to aliquots of the whole blood. In the second group of patients, febrile phase plasmas evoked HLM depression, figures 6 and 7, however, further significant depression was elicited by the addition of 0.1-1.0 ng exogenous endotoxin per ml whole blood, which is incompatible with the initial inhibition being mediated by endogenous endotoxin. That such depression of HLM was mediated by non-endotoxin factors was further supported by the findings that in patients V, VI, VII, VIII and X, figures 6 and 7, the afebrile phase plasmas also depressed HLM significantly. Moreover, the addition of 0.1-1.0 ng exogenous endotoxin per ml blood resulted in further significant HLM depression, demonstrating that when HLM depression by test plasma is relatable to non-endotoxin depression, exogenous endotoxin is fully capable of augmenting such depression. Although 0.1 ng/ml endotoxin concentration was capable of producing significant HLM inhibitory activity in the plasma of most of the 13 patients, it is of interest to note that in patients VII, VIII, and X no significant depression was produced by this concentration in both the febrile and afebrile plasmas; rather in these patients 1.0 ng/ml endotoxin was required to evoke significant depression in both sets of plasmas. The reason for such lowered detectability of endotoxin in certain plasmas is not presently known. Thus, in none of the assays in 13 highly febrile patients with typhoid fever was there a response combatible with the presence of circulating endotoxin despite an assay sensitivity as low as 0.1-1.0 ng endotoxin per ml blood.

DISCUSSION

Although inhibition of HLM by endotoxin has been previously described, the lower limits of such inhibition have not been critically evaluated. In 1952, Martin and Chaudhuri, using a slide cell technique, demonstrated that purified bacterial products from several gram-negative bacteria were potent inhibitors of HLM. As little as 5.0 ng/ml of a meningococcal filtrate evoked inhibition (1). Berthrong and Cluff, however, using rabbit leukocytes in a slide cell method, were unable to demonstrate inhibition of leukocyte migration *in vitro* employing *Serratia marcescens* endotoxin in concentrations as high as 5,000 to 25,000 ng/ml plasma (7). Nevertheless, Watanabe and Tanaka reported *in vitro* inhibition of rabbit leukocyte migration by *Shigella flexneri* and *Salmonella paratyphi* endotoxins at 10,000 ng/ml blood. *In vivo*, these endotoxins inhibited migration at concentrations estimated as low as 3 ng/ml rabbit blood (8). Most recently, Bryant et al (2) reported that *E. coli* endotoxin at concentrations of 1,000 ng/ml blood clearly produced HLM depression *in vitro*, but did not extend their observations below this concentration. The present studies confirm and extend these latter observations. The data indicate that: 1) HLM is significantly depressed by concentrations as low as .05 ng endotoxin per ml assay suspension, 2) human plasmas obtained from blood to which exogenous endotoxin has been added can evoke HLM depression, and such depression is detectable with concentrations as low as 0.1-1.0 ng endotoxin per ml blood; and 3) progressive significant decrements of HLM occur with each ten-fold increment in endotoxin concentration. The mechanism by which endotoxin inhibits HLM has been attributed to non-specific cellular aggregation and adherence to glass as a general feature of phagocytosis (2), or may be due to a neutrophil inhibition factor elaborated after incubation

with endotoxin (9).

Most of the available assays for endotoxin do not possess the sensitivity of the HLM bioassay (10-12). The few assays which do possess comparable sensitivity have not been adapted for endotoxin assay of clinical materials (13-14). Only the limulus lysate method of Levin et al (15) and Rienhold and Fine (16) have comparable sensitivity (0.5 ng/ml blood); however, the specificity of this method for endotoxin detection has recently been questioned (17). Indeed, recent data indicate that the assay is not specific for endotoxin becoming positive in the presence of thrombin, thromboplastin, ribonuclease, and certain polynucleotides (18). The HLM bioassay as performed by the methods described above can detect 0.1-1.0 mg exogenous endotoxin per ml blood. Moreover, inhibition of HLM appears fairly specific for endotoxin. Thus, Bryant et al (4), concluded from their studies that HLM was resistant to wide variations in glucose, electrolytes and pH, but was dependent on physiologic ranges of magnesium concentration. High concentrations of staphylococci, either living or dead (1:5 leukocytes and 1:10 leukocytes, respectively), and of latex particles (mean size 0.81 mu) at concentrations of 2/leukocyte inhibited HLM. Recently Bryant demonstrated that hypertonic sucrose, urea, and saline were inhibitory to HLM; however, in the physiologic ranges (below 400 milliosmoles/kg) only sucrose was inhibitory (19). Of several steroids studied by Ketchel et al (6) only hydrocortisone was found to be inhibitory to HLM at concentrations of 10 mcg/ml. Endogenous hydrocortisone, however, would be unlikely to affect HLM as peak levels in normal man (20) are at least 1/100th of the amount demonstrated by Ketchel et al to produce inhibition. Furthermore, the present HLM assay system results in a twenty-fold dilution of test plasma so that only concentrations of hydrocortisone that are 2,000

times greater than peak concentrations in normal man would be predicted to produce significant HLM depression. Watanabe and Tanaka (9) found that capillary tube migration of rabbit leukocytes was unaffected by epinephrine, norepinephrine, serotonin, ACTH, corticosterone, reserpine, atropine, dihydroergotamine, chlorpromazine, and LSD in the concentrations studied. Thus there is a body of evidence to suggest that HLM is not impaired by a wide range of physiologic and pharmacologic alterations.

In the present study of 13 highly febrile typhoid fever patients, no plasma was positive for endotoxin by the required criteria, suggesting that the level of endotoxemia in this disease is below the limits of sensitivity of the present assay, i.e. less than 0.1-1.0 ng/ml blood. It is unlikely that the plasma endotoxin inactivator described by Keene et al (21) played a role in these negative assays since all plasmas were manipulated at temperatures at which the activity of this inactivator is negligible; and since the additional control studies utilizing trace quantities of exogenous endotoxin evoked detectable inhibitory effects.

The HLM bioassay as presently described requires 6-7 hours to perform. The numerous procedures and time consuming nature of the HLM bioassay compare unfavorably with the relatively simple limulus assay (15). Nevertheless, the HLM bioassay is very sensitive; and with further investigation of its properties and the development of an endotoxin extraction method from plasma to further enhance its sensitivity, it should prove a useful method for the study of endotoxemia.

Finally, the potent effect of endotoxin in inhibiting human leukocyte migration raises the possibility of this activity exerting a significant deleterious effect on host resistance to microbial pathogens in vivo. The well known ability of endotoxemia to reduce host resist-

ance to a variety of pathogens early after its administration has been postulated to result, in part at least, from host inability to mobilize granulocytes; the latter effect has been attributed primarily to the granulocytopenic activity of endotoxin (22). The present findings indicate that such reduction in leukocyte mobilization at the sites of infection during endotoxemia may also be based upon inhibition of leukocytic migratory activity.

REFERENCES

1. Martin, S. F. and Chaudhuri, S. N. Effect of bacterial products on migration of leukocytes. Proc. Soc. Exp. Biol. Med. 81:286-288, 1952.

2. Bryant, R. E., Des Prez, R. M. and Rogers, D. E. Studies on human leukocyte motility. II. Effects of bacterial endotoxin on leukocyte migration, adhesiveness, and aggregation. Yale J. Biol. Med. 40:192-204, 1967.

3. Ketchel, M. M. and Favour, C. B. The accleration and inhibition of migration of human leukocytes in vitro by plasma protein fractions. J. Exp. Med. 101: 647-663, 1955.

4. Bryant, R. E., Des Prez, R. M., Van Way, M. H., and Rogers, D. E. Studies on human leukocyte motility. I. Effects of alterations in pH, electrolyte concentration, and phagocytosis on leukocyte migration, adhesiveness, and aggregation. J. Exp. Med. 124:483-499, 1966.

5. Nahas, G. G., Tannieres, M. L. and Lennon, J. F. Direct measurement of leukocyte motility: effects of pH and temperature. Proc. Soc. Exp. Biol. Med. 138:350-352, 1971.

6. Ketchel, M. M., Favour, C. B. and Sturgis, S. H. The in vitro action of hydrocortisone on leukocyte migration. J. Exp. Med. 107:211-218, 1958.

7. Berthrong, M. and Cluff, L. E. Studies of the effect of bacterial endotoxins on rabbit leukocyte. I. Effect of intravenous injection of the substances with and without induction of the local Schwartzman reaction. J. Exp. Med. 98:331-347, 1953.

8. Watanabe, T. and Tanaka, F. Studies on the inhibitory effects of endotoxin upon leukocyte migration. Japan. J. Exp. Med. 30:449-474, 1960.

9. Goetzl, E. J. and Austen, K. F. A neutrophil immobilizing factor derived from human leukocytes. I. Generation and partial characterization. J. Exp. Med. 136: 1564-1580, 1972.

10. Porter, P. J., Spievack, A. R., and Kass, E. H. Endotoxin-like activity of serum from patients with severe localized infections. N. Engl. J. Med. 271:445-447, 1964.

11. Filkins, J. P. Bioassay of endotoxin inactivation in the lead-sensitized rat. Proc. Soc. Exp. Biol. and Med. 134:610-612, 1970.

12. Baker, P. J. and Wilson, J. B. Hypoferremia in mice and its application to the bioassay of endotoxin. J. Bacteriol. 90:903-910, 1965.

13. Heilman, D. H. and Bast, R. C. In vitro assay of endotoxin by the inhibition of macrophage migration. J. Bacteriol. 93:15-20, 1967.

14. Pieroni, R. E., Broderick, E. J., Bundeally, A., and Levin, L. A simple method for the quantification of submicrogram amounts of bacterial endotoxin. Proc. Soc. Exp. Biol. Med. 133:790-794, 1970.

15. Levin, J., Tomasulo, P. A. and Oser, R. S. Detection of endotoxin in human blood and demonstration of an inhibitor. J. Lab. Clin. Med. 75:903-911, 1970.

16. Reinhold, R. B. and Fine, J. A technique for quantitative measurement of endotoxin in human plasma. Proc. Soc. Exp. Biol. Med. 137:334-339, 1971.

17. Stumacher, R. J., Kovnat, M. J., and McCabe, W. R. Limitations of the usefulness of the limulus assay for endotoxin. N. Engl. J. Med. 288:1261-1264, 1973.

18. Elin, R. J. and Wolff, S. M. Studies of the specificity of the limulus amoebocyte lysate test. Abstracts of the Annual Meeting of the American Society of Microbiology, 1973, Abst M180, p. 103.

19. Bryant, R. E., Sutcliffe, M. C., and McGee, Z. A. Effect of osmolalities comparable to those of the renal medulla on function of human polymorphonuclear leukocytes. J. Infect. Dis. 126:1-10, 1972.

20. Braunsberg, H. and James, B. H. The determination of adrenocortical steroids in blood. Results in normal individuals and adrenal hyperfunction. J. Endocr. 21:327-340, 1960.

21. Keene, W. R., Landy, M. and Shear, M. J. Inactivation of endotoxin by a humoral component. VII. Enzymatic degradation of endotoxin by blood plasma. J. Clin. Invest. 40:302-310, 1961.

22. Mulholland, J. H. and Cluff, L. E. The effect of endotoxin upon susceptibility to infection: the role of the granulocyte. Bacterial Endotoxins M. Landy and W. Braun, editors, 1964, pp. 211-229.

LEGENDS

Figure 1. Typical effect of varying concentrations of S. typhimurium endotoxin on normal human leukocyte migration (HLM).

Figure 2. Sensitivity of detection of HLM inhibitory activity in plasma obtained from whole human blood to which S. typhimurium endotoxin has been added in vitro.

Figure 3. Evidence that two-fold increments in endotoxin concentration in whole human blood does not result in significant increments in ability of plasma to depress HLM; in contrast, ten-fold increments in endotoxin concentrations do significantly further augment HLM inhibitory activity of plasma.

Figure 4A. Comparative effects of S. typhimurium and S. typhosa endotoxins on HLM.

Figure 4B. Effect of combining S. typhosa and S. typhimurium endotoxins at concentrations with identical HLM inhibitory activity. Note the lack of detectable augmentation of inhibition.

Figure 5. Assays of three highly febrile typhoid fever patients whose test plasmas (TP) did not depress HLM when compared with controls (C).

Figures 6 and 7. Assays of ten highly febrile typhoid fever patients whose test plasmas (TP) when compared with controls (C) inhibited HLM; however, test plasmas from aliquots of the same blood to which 0.1-1.0 ng/ml exogenous endotoxin had been added (TP + 0.1-1.0E) did produce a further significant depression of HLM. See text for the significance of these findings.

MECHANISMS OF ENDOTOXIN TOLERANCE

IX. EFFECT OF EARLY EXCHANGE TRANSFUSION ON ENDOTOXIN LETHALITY*

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Running title: Mechanisms of endotoxin tolerance

When gram-negative bacterial endotoxins are administered intravenously to healthy man or animal, they are cleared from the circulation in exponential fashion. The initial clearance phase is rapid, with a slower phase generally becoming evident within 30 minutes (1-3). The major contribution of the reticuloendothelial system (RES) to such endotoxin clearance has been documented previously (1, 2). When tolerance to endotoxin is induced by prior endotoxin injections, the rate, as well as total RES uptake of circulating toxin become markedly enhanced (2-5). Based upon such findings, together with the apparent abolition of tolerance following RES "blockade", Beeson proposed that enhanced RES removal of circulating endotoxin mediates tolerance; this mechanism presumably would act by protecting other more susceptible tissues from toxin injury (5). This attractive hypothesis, however, has been challenged by a number of recent studies (2, 6-10). The significance of this challenge is of more than academic interest, since it is now feasible to enhance removal of circulating endotoxin by physical means, e.g. exchange transfusion. Thus, following the introduction of a lethal dose of endotoxin into the bloodstream, assistance of the normal (i.e., non-tolerant) RES with toxin removal by early and rapid exchange transfusion can simulate the enhanced blood clearance accomplished by the RES in tolerant animals. Significant protection should now result if enhanced blood clearance per se is indeed the basis of tolerance. The present studies were designed to test this possibility.

Materials and Methods. A Boivin preparation of Escherichia coli endotoxin, 0127B8 (Difco) was labelled with $NaCr^{51}O_4$ (Abbott Laboratories) by the method of Braude and coworkers (11). Control solutions of $NaCr^{51}O_4$ were treated identically except for omission

of endotoxin to determine the quanitity of $NaCr^{51}O_4$ that remained non-dialyzable as a result of spontaneous aggregation under the conditions of labeling. It was found that a maximum of only 1% of the labelled E. coli endotoxin preparation could be contaminated with unbound $Cr^{51}O_4$ ion under the conditions of the labeling.

Employing aseptci precations, one femoral artery of 2.0 to 2.5 kg healthy albino New Zealand rabbits was cannulated with sterile polyethylene tubing (PE 90, Clay Adams, Inc.) previously rinsed repeatedly with pyrogen-free sterile saline. An LD_{80} dose of unlabelled E. coli endotoxin, 2500 ug, as determined in a control group of 10 untreated animals observed for 96 hours, was then administered via ear vein. Twenty minutes later, an exchange transfusion was carried out as follows: 10 ml blood was rapidly withdrawn via the femoral artery cannula and 10 ml of freshly drawn, heparinized blood from healthy rabbit donors immediately returned through the same cannula by turning a three way stopcock. The donor blood was pretreated at room temperature (70-72 °F) by filtering through sterile, pyrogen-free polyethylene screens of the type used in human transfusion sets to remove microthrombi. Additional 10 ml aliquots of blood were exchanged repeatedly until the recipient had received the equivalent of 15% body weight of donor blood (280 to 350 ml). This accomplished the exchange of approximately 80% of the recipients initial blood volume as determined by plasma protein labeling studies with Evans Blue dye. The entire exchange transfusion was always completed within 20 minutes. Randomly selectec control animals were concomitantly given 2500 ug of the E. coli endotoxin by ear vein, and 20 minutes later sham exchange transfusion performed, i.e., repeated, 10 ml aliquots of blood were rapidly withdrawn via a femoral artery cannula and returned to the same animal after addition of sterile,

pyrogen-free heparin equivalent to that utilized in the actual exchange transfusion (4000 U.S.P. units). Following either the exchange or sham exchange procedure, the femoral artery cannula was removed, the artery ligated, and the wound closed with sterile sutures. All animals were observed for 96 hour survival. Additional control studies were carried out to determine whether exchange transfusion per se enhanced susceptibility to endotoxin lethality. For this purpose, femoral artery cannulation was performed in a group of 10 rabbits. Five animals were not exchange transfused but were given 4000 U.S.P. units heparin intravenously, the other 5 animals were exchange transfused with blood from healthy donors as described above. The cannulas were removed, the femoral artery ligated, and the femoral wound sutured with sterile precautions. All animals were then given an LD_{20} dose of the E. coli endotoxin (500 ug) via ear vein, and 96 hour survivals monitored.

Blood clearance studies of the E. coli endotoxin were performed in 3 groups of animals - normal, normal exchange transfused, and tolerant. The tolerant rabbits were studied on day 8 following 7 daily intravenous injections of 100 ug unlabelled E. coli endotoxin. In preliminary studies, 10 animals thus pretreated were found highly tolemnt, i.e. exhibited no mortality after ear vein administration of 2500 ug unlabelled E. coli endotoxin. The femoral artery of each test animal was cannulated, and 2500 ug Cr^{51}-tagged E. coli endotoxin injected via ear vein. At carefully timed intervals, 1 ml blood samples were removed from the femoral artery cannula qnd discarded (washout), and a second 1 ml sample removed and placed in plastic tubes of uniform size. Radioactivity was determined by counting in an automatic gamma well counter for sufficient time to permit reproducibility to within 5%. The amount of circulating endotoxin was expressed as the percentage of administered dose

of radioactivity calculated to be present at each time interval in the total blood volume of each animal.

Results. Ear vein administration of 2500 ug Cr^{51}-tagged E. coli endotoxin into healthy, non-tolerant rabbits resulted in typical blood clearance patterns, i.e. an initial rapid phase followed within 30 minutes by the slower phase. This latter phase was characterized by prolonged circulation of appreciable quantities of the initially injected dose of toxin- between 20 to 30%, Figure 1 (curve 1). In contrast, when the tagged toxin was injected into endotoxin tolerant animals, the expected marked enhancement in blood clearance was seen, Figure 1 (curve 3). When exchange transfusion was carried out in non-tolerant animals, the circulating endotoxin was rapidly and permanently reduced to levels closely approximating those in tolerant animals, Figure 1 (curve 2).

Figure 2A demonstrates that exchange transfusion did not per se significantly enhance susceptibility to endotoxin. Thus, a 20% mortality occurred when 500 ug of the E. coli endotoxin was administered after exchange transfusion, identical to the mortality of comparably cannulated and heparinized but non-exchanged control animals. Figure 2B demonstrates that early exchange transfusion did not significantly protect against endotoxin challenge. Thus, when 2500 ug E. coli endotoxin was administered and 20 minutes later exchange transfusion performed, 96 hour mortality was only slightly, and not significantly reduced, being 70% compared to 83% in control animals sham exchanged with their own blood.[1] This latter figure also further demonstrates that exchange transfusion per se did not enhance endotoxin susceptibility, since 2500 ug E. coli endotoxin represented an LD_{80} in control non-exchanged animals.

Discussion. By means of exchange transfusion begun 20 minutes following intravenous injection of an LD_{80} bolus of E. coli endotoxin into healthy non-tolerant rabbits, it was possible to minimize the high levels of endotoxin that otherwise continue to circulate for hours. The 20 minute interval between endotoxin injection and exchange transfusion was carefully selected so as to permit appreciable RES uptake of endotoxin followed by rapid removal of residual circulating toxin, thereby simulating the enhanced clearance of circulating endotoxin that is characteristic of the tolerant state. It is emphasized that the enhanced rate of clearance of endotoxemia achieved by such exchange transfusion did not precisely duplicate that seen in the tolerant animal, since exchange was deliberately not begun until 20 minutes after toxin injection to allow the RES to remove at least 50% of the endotoxin. Nevertheless, the resulting final endotoxin blood levels, as well as the time at which such levels attained minimal values after exchange transfusion, were virtually identical to that in the tolerant animal. Despite this ability of exchange transfusion to elicit rapid and sustained reduction of endoroxemia, only slight and statistically insignificant reduction in the subsequent 96 hour mortality ensued. This contrasted with the xero mortality in the tolerant animals. This apparent lack of protection by exchange transfusion could not be related to endotoxin susceptibility secondary to the exchange procedure per se.

Earlier studies have shown continued activity of the endotoxin tolerant mechanisms despite RES "blockade" (6), enhanced, rather than reduced, endotoxin susceptibility after stimulation of RES phagocytic activity with zymosan, BCG, triolein, or glucan (2, 7-10), lapse of tolerance following discontinuance of endotoxin injections despite persistence of accelerated blood clearance of toxin (12), absence of accelerated endotoxin clearance

during the early phase of tolerance (13), toxicity of rough endotoxins despite their rapid blood clearance (14), and enhanced resistance of the reticuloendothelial cells of tolerant animals to endotoxin toxicity both in vitro (15) and in vivo (16). Considered collectively, these earlier findings demonstrated that mechanisms other than enhanced RES phagocytic activity are key determinants of the endotoxin tolerant state. The present findings directly confirm this thesis and support the alternative concept developed during studies on lysosomal disruption and on pyrogenic tolerance to endotoxin, i.e., that tolerance is based primarily upon refractoriness of the highly susceptible RES to endotoxin injury, and that accelerated clearance represents only an ancillary protective mechanism in that toxin is brought more rapidly into these refractory cells (16, 17).

Summary. Healthy New Zealand rabbits were injected intravenously with an LD_{80} dose of E. coli endotoxin. Twenty minutes later, after greater than 50% removal of the endotoxin by the RES, an exchange transfusion was performed, accomplishing a rapid and sustained reduction in the level of endotoxemia approximating that in animals rendered highly tolerant by 7 prior sublethal injections of toxin. Despite such reduction in endotoxemia, the 96 hour mortality was only slightly, and not significantly, reduced compared to sham exchanged controls (70 vs 83%, respectively). Control studies indicated that the exchange transfusion per se did not enhance endotoxin mortality. The findings directly support the concept that endotoxin tolerance is based primarily upon enhanced RES resistance to the toxicity of endotoxin rather than upon enhanced RES clearance of circulating endotoxin.

REFERENCES

1. Braude, A.I., Carey, F.J., and Zalesky, M., J. Clin. Invest. 34, 858 (1955).

2. DiLuzio, N.R. and Crafton, C.G., Proc. Soc. Exp. Biol. Med. 132, 686 (1969).

3. Greisman, S.E., Hornick, R.B., Wagner, H.N. Jr., Woodward, W.E., and Woodward, T.E., J. Clin. Invest. 48, 613 (1969).

4. Carey, F.J., Braude, A.I., and Zalesky, M., J. Clin. Invest. 37, 441 (1958).

5. Beeson, P.B., J. Exp. Med. 86, 39 (1947).

6. Greisman, S.E., Carozza, F.A. Jr., and Hills, J.D., J. Exp. Med. 117, 663 (1963).

7. Benacerraf, B.G., Thorbecke, J., and Jacoby, D., Proc. Soc. Exp. Biol. Med. 100, 796 (1959).

8. Suter, E., Ullman, E.G., and Hoffman, R.G., Proc. Soc. Exp. Biol. Med. 99, 167 (1958).

9. Stuart, A.E., and Cooper, G. N., J. Path. Bact. 83, 245 (1962).

10. Lemperle, G., Proc. Soc. Exp. Biol. Med. 122, 1012 (1966).

11. Braude, A.I., Carey, F.J., Suterhland, D., and Zalesky, M., J. Clin. Invest. 34, 850 (1955).

12. Atkins, E. and Wood, W.B. Jr., J. Exp. Med. 101, 519 (1955).

13. Braude, A.I., Zalesky, M., and Douglas, H., J. Clin. Invest. 37, 880 (1966).

14. Chedid, L., Parant, F., Parant, M., and Boyer, F., N.Y. Acad. Sci. 133, 712 (1966).

15. Dinarello, C.A., Bodel, P.T., and Atkins, E., Trans. Assoc. Am. Physcns. 81, 334 (1968).

16. Weissman, G. and Thomas, L., J. Exp. Med. 116, 433 (1962).

17. Greisman, S.E. and Hornick, R.B., J. Infect. Dis. 128 (Supplement), 265 (1973).

FOOTNOTES

[1] The observed difference in percentage mortality (13%) is less than twice its standard error (12.4).

LEGEND

Fig. 1. Blood clearance of 2500 ug Cr^{51}-tagged E. coli endotoxin in normal (curve 1), normal exchange transfused (curve 2), and tolerant (curve 3) rabbits. Note the prolonged circulation of high concentrations of endotoxin in normal animals and the ability of exchange transfusion to simulate the endotoxin clearance curve of the tolerant animal.

Fig. 2. Effect of exchange transfusion on E. coli endotoxin 96 hour mortality. Exchange resulted in no enhancement of susceptibility to endotoxin (Panel A), and only slight and statistically insignificant reduction in endotoxin mortality (Panel B).